Responses of Drosophila giant descending neurons to visual and mechanical stimuli

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RESEARCH ARTICLE

Responses of *Drosophila* giant descending neurons to visual and mechanical stimuli

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ABSTRACT

In *Drosophila*, the paired giant descending neurons (GDNs), also known as giant fibers, and the paired giant antennal mechanosensory descending neurons (GAMDNs), are supplied by visual and mechanosensory inputs. Both neurons have the largest cell bodies in the brain and both supply slender axons to the neck connective. The GDN axon thereafter widens to become the largest axon in the thoracic ganglia, supplying information to leg extensor and wing depressor muscles. The GAMDN axon remains slender, interacting with other descending neuron axons medially. GDN and GAMDN dendrites are partitioned to receive inputs from antennal mechanosensory afferents and inputs from the optic lobes. Although GDN anatomy has been well studied in *Musca domestica*, less is known about the *Drosophila* homolog, including electrophysiological responses to sensory stimuli. Here we provide detailed anatomical comparisons of the GDN and the GAMDN, characterizing their sensory inputs. The GDN showed responses to light-on and light-off stimuli, expanding stimuli that result in luminance decrease, mechanical stimulation of the antennae, and combined mechanical and visual stimulation. We show that ensembles of lobula columnar neurons (type Col A) and mechanosensory antennal afferents are likely responsible for these responses. The reluctance of the GDN to spike in response to stimulation confirms observations of the *Musca* GDN. That this reluctance may be a unique property of the GDN is suggested by comparisons with the GAMDN, in which action potentials are readily elicited by mechanical and visual stimuli. The results are discussed in the context of descending pathways involved in multimodal integration and escape responses.

KEY WORDS: Giant descending neurons, Giant fiber, Antennal mechanosensory region, Lobula, Multimodal integration, *Drosophila*, Whole-cell patch clamp

INTRODUCTION

Escape behaviors in insects consist of stereotyped locomotory sequences that are initiated by sensory inputs and controlled by local neural circuits in the thoracic ganglia. In insects, signals declarative of threatening situations are commonly relayed by specialized sensory and interneuronal pathways (Tauber and Camhi, 1995). Although studies of escape behaviors have almost uniformly employed unimodal stimuli (see Card, 2012), neuroanatomical and electrophysiological studies of descending neurons in Diptera demonstrate that the brain integrates a variety of cephalic sensory inputs, including visual, olfactory, mechanical and ascending information from the thorax and abdomen (Strausfeld and Bacon, 1983; Gronenberg and Strausfeld, 1990). Descending neurons convey integrated sensory information from the brain to the thoracic ganglia, where they activate thoracic motor centers, such as those mediating stabilized flight, voluntary actions or escape behaviors. In flies, the pair of giant descending neurons (GDNs), also referred to as giant fibers, is responsible for light-off initiated escape behavior in white-eyed *Drosophila* mutants (Levine, 1974; Thomas and Wyman, 1984; Trimarchi and Schneiderman, 1995). Direct photostimulation of the ionotropic purinoceptor $P2X_2$ in genetically targeted GDNs in red-eyed flies can also initiate escape behavior (Lima and Miesenböck, 2005). Wild-type *Drosophila* also escape in response to visual looming stimuli (Hammond and O'Shea, 2007), but this appears to be mediated by another neural pathway because the detailed locomotory sequence in escapes initiated by light-off is different from that of looming-initiated escape (Card and Dickinson, 2008a; Card and Dickinson, 2008b). Nor do the GDNs appear to be activated by a looming stimulus (Fotowat et al., 2009). Likewise, in *Musca*, a second flight initiation pathway, distinct from that mediated by the GDN, elicits escape responses to looming stimuli (Holmqvist, 1994). Comparative studies on larger dipteran species (*Musca domestica* and *Calliphora erythrocephala*) yielded important data about the similarities of GDN responses with those of *Drosophila*: visual stimuli (light on and off, small-field motion) and mechanical stimulation of the antenna are all able to initiate subthreshold activity (Bacon and Strausfeld, 1986). This is consistent with the demonstration of cobalt coupling between specific dendritic GDN subfields and antennal mechanosensory afferents, and visual afferents originating in the lobula (Bacon and Strausfeld, 1986). The GDN makes an electrical synaptic connection with the tergotrochanteral muscle ('jump' muscle) motor neuron (TTMn) (King and Wyman, 1980), again consistent with cobalt coupling between the GDN and the TTMn (Bacon and Strausfeld, 1986). The peripherally synapsing interneuron (PSI), which establishes chemical synapses onto the axons of wing-depressor motor neurons, is likewise cobalt and dye coupled.

The first suggestion of an escape pathway in response to a visual looming stimulus not involving the GDN or TTMn was from observations of *Musca domestica* (Holmqvist, 1994). Descending neurons (DNs) mediating this alternative escape pathway have not yet been identified. Possible candidates are other members of the GDN cluster that comprise a system of at least eight parallel channels, which with the GDN likely share at least the same lobula-derived inputs (Strausfeld and Bacon, 1983; Milde and Strausfeld, 1990). More recent studies on the *Drosophila* GDN have shown that looming visual stimuli elicit subthreshold responses (Mu et al., 2012a), as do acoustic stimuli detected by the antenna's Johnston's organ (Tootoonian et al., 2012) or direct mechanical stimulation of the antenna (Lehnert et al., 2013). These subthreshold GDN responses were elicited in restrained flies, but in a preparation in which the

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List of abbreviations

AL	antennal lobe
AMMC	antennal mechanosensory and motor center
CA	calyx
DN	descending neuron
EB	ellipsoid body
EPSP	excitatory post-synaptic potential
FB	fan-shaped body
GAMDN	giant antennal mechanosensory descending neuron
GCI	giant commissural interneuron
GDN	giant descending neuron
GFP	green fluorescent protein
LAL	lateral accessory lobe
LH	lateral horn
LOP	lobula plate
METH	metathoracic ganglion
MTH	mesothoracic ganglion
NC	neck connective
PSI	peripherally synapsing interneuron
PTH	prothoracic ganglion
PVLP	posterior ventral protocerebrum
SLP	superior lateral protocerebrum
TTMn	tergotrochanteral motor neuron

mesothoracic legs were left free to elicit the escape jump, a looming stimulus did initiate single GDN spikes on some occasions (Von Reyn and Card, 2012). This suggests that thoracic sensory inputs may have facilitating effects on the GDN–TTMn circuit. However, what has not been determined is whether the GDN's usual reluctance to spike might be an important aspect of its natural physiology.

To attempt to resolve this, we report a more detailed anatomical and physiological study of the GDN, including presentation of bimodal stimuli and its comparison with a second type of giant neuron that spikes readily. While it is difficult to repetitively collect neurophysiological data from the GDN in larger flies using conventional intracellular recordings, developed genetic tools for *Drosophila* providing the expression of green fluorescent protein (GFP) in the cerebral neurons allows specific targeting of the GDN and other DNs for whole-cell patch clamp recording.

By employing these tools, we demonstrate convergence of lobula columnar (type Col A) visual neuron afferents with at least two other lobula outputs onto three dendritic branches of the GDN within the brain's protocerebrum. We show antennal mechanosensory afferents from Johnston's organ supplying a third dendritic branch that extends rostrally into the deutocerebrum. Patch-clamp recordings show that the GDN responds inconsistently to light-on, but more reliably to light-off stimuli. Expanding darkening stimuli across the retina results in a luminance decrease and subthreshold responses, as does mechanical stimulation of the antenna. Coincident bimodal stimulation of the GDN elicits larger subthreshold responses.

Comparing the physiological properties of the GDN with those of other DNs (Mu et al., 2012b), such as the giant antennal mechanosensory descending neuron (GAMDN), demonstrates that other neurons with cell bodies and dendritic arborization of similar size indeed spike reliably in response to similar visual and mechanical stimuli. Spiking responses by other DNs in restrained preparations accentuate the reluctance of the GDN to spike.

RESULTS

Multimodal inputs to the GDN

Circuit elements

Streptavidin:Cy3 was used to confirm the identity of the GDN (Fig. 1A,B), which was injected with biocytin during recording. This precaution was taken because GFP is not exclusively expressed in GDNs in the Gal 4 lines used. Furthermore, dye injection also usefully results in transsynaptic fills of the contralateral giant commissural interneurons (GCIs) that connect the left and right ventro-lateral protocerebrum (Fig. 1A). Their labeling, together with that of the GDN, is consistent with previous results in *Drosophila* (Phelan et al., 1996; Mu et al., 2012a) and *Musca* (Bacon and Strausfeld, 1986).

Similar to the GDN anatomy in larger fly species, the *Drosophila* GDN has four major dendritic domains, three in the protocerebrum and a fourth extending caudally into the antennal mechanosensory and motor center of the deutocerebrum (numbered 1–4 in Fig. 1C). Dendritic subfield 3 and the proximal part of subfield 4 are coupled to the axon terminals of Col A neurons (Fig. 1C,D). The caudal (deutocerebral) portion of subfield 4 receives a subset of sensory afferent from Johnston's organ (Fig. 1E). These are part of a massive supply of sensory neuron terminals into the antennal mechanosensory and motor center (AMMC). Their terminals also extend into ipsilateral dendrites and axon collaterals from the GAMDN (see below).

Two giant commissural interneurons are linked to the GDN's dendritic subfield 1 (Fig. 1F) and these interneurons are also resolved as contiguous with Col A terminals (Fig. 1G,H). Thus, Col A neurons provide outputs to the ipsilateral GDN and the GCIs, the latter coupled to both the ipsilateral and contralateral GDNs.

Gal 4 lines demonstrate that although Col A and Johnston's organ afferents are the most prominent visual and mechanical sensory inputs to the GDN, respectively, other visual neurons must play a role in GDN activity. Two of these are shown in Fig. 1C,D, both resolved as single and quite large-diameter axons extending to subfield 2 from the lobula. Nor are Col A neurons the sole output from the lobula. To date, approximately 18 morphologically distinct ensembles of lobula neurons have been shown to target discrete neuropils, called optic glomeruli, distributed in the lateral protocerebrum (Otsuna and Ito, 2006). Each lobula ensemble provides discrete bundles of axons to this part of the brain (arrowed in Fig. 1H). Two of the rostro-ventral optic glomeruli supply dendrites of the GAMDN, as described below.

Responses

As in larger fly species (Bacon and Strausfeld, 1986), current injection initiated action potentials in the GDN (Fig. 2A). However, none of the visual and mechanical stimuli used in our experiments initiated action potentials. Instead, such stimulation resulted in sub-threshold depolarization or hyperpolarization (Figs 2–4). The reluctance of the GDN to spike was not due to the patch-clamp recording technique employed because other identified DNs can be routinely recorded in the same preparations, and these respond with action potentials to unimodal stimuli (Figs 5, 6) (Mu et al., 2012b).

Responses of the GDN to light-on and -off stimuli

A light-off stimulus generally elicited an excitatory post-synaptic potential (EPSP) in the GDN whereas the responses to a light-on stimulus were more variable (Fig. 2B,C). Fig. 2C shows examples of three different responses to the same light-on stimulus in one preparation: depolarization, hyperpolarization and no obvious response. The strength of the response to light-off was related to the duration of the preceding period of light-on (Fig. 2D), but the effect began to plateau if the preceding light-on condition lasted longer than 10–15 s. The effects of the preceding light-on duration on the light-off response were consistent across different preparations. Fig. 2D shows that the responses to light-off

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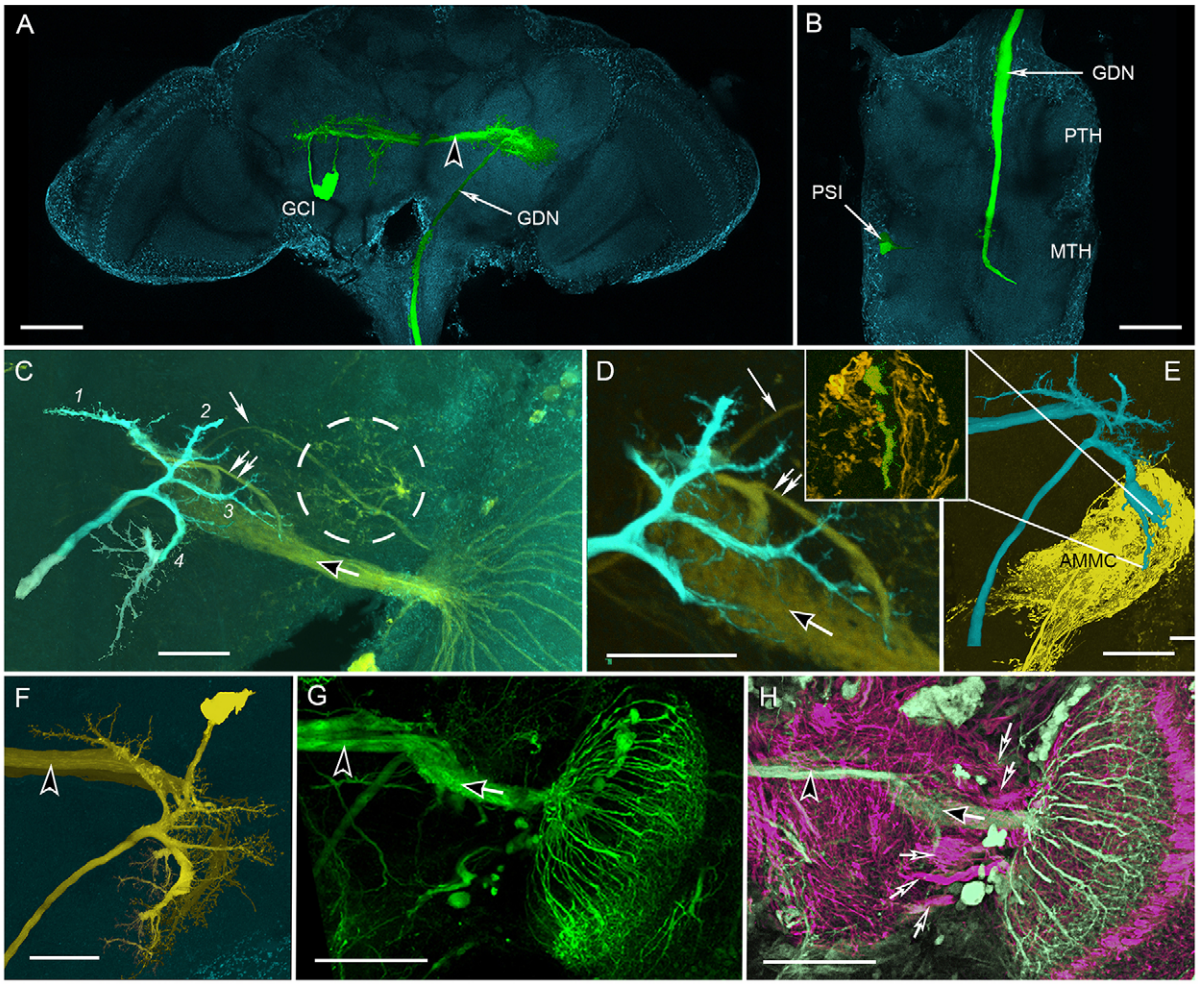

Fig. 1. Neural organization associated with the giant descending neuron (GDN; giant fiber). (A,B) Scanning confocal micrographs of the GDN in the brain (A) and prothoracic (PTH) and mesothoracic ganglion (MTH; B), showing its most obvious dye coupling with paired contralateral giant commissural interneurons (GCI) and, in the MTH, with the peripherally synapsing interneurons (PSI) and tergotrochanteral muscle motor neuron (pale cell body adjacent to PSI). Note the narrow diameter of the GDN axon in the brain and its expansion in the neck connective and thoracic ganglia. (C) Scanning confocal micrograph showing Col A axons (direction indicated by black arrow) terminating onto GDN dendritic branches 1, 3 and 4. The clustered terminals denote the Col A optic glomerulus. A second glomerulus (ringed) is occupied by terminals of another type of lobula columnar neuron that has no relationship to the GDN. In addition to Col A neurons, two other prominent axons (white arrows) extend from the optic lobe to the GDN dendrites. (D) Enlargement showing the segregation of optic lobe afferents to different parts of the GDN tree. (E) Dye-filled sensory neuron terminals occupy most of the antennal mechanosensory and motor center (AMMC), with some enwrapping the GDN's deutocerebral dendritic tree 4 (see inset). (F) Dye coupling between the GDN and GCIs (arrowhead). (G) Contiguity between the axons of Col A neurons (triangular arrow) and the GCIs (arrowhead). (H) The same relationship as in G, but showing neuroarchitecture revealed by anti-β-tubulin. Small arrows indicate numerous axons bundles from the lobula en route to their relevant optic glomeruli in the lateral protocerebrum. Upper bundles extend to glomeruli receiving dendritic branches of the GAMDN shown in Fig. 5. Scale bars: (A,B,G,H) 50 µm; (C–F) 25 µm.

occurring 15 s after the light-on condition were significantly larger than those 1 and 5 s after the light-on condition (ANOVA, $P<0.05$, $N=8$ animals, 40 trials for 1 s, 38 trials for 5 s, 37 trials for 10 s, 55 trials for 15 s).

When stimulated with a slow 0.5 Hz flicker, the GDN usually rapidly adapted with only the first on and off phases able to initiate clear responses (Fig. 2E, top trace). The middle trace in Fig. 2E shows an exceptional example of responses to slow flicker, in which the second and third cycles of flicker also elicited similar responses to that of the first flicker cycle. A 10 Hz flicker stimulus did not initiate noticeable EPSPs or inhibitory post-synaptic potentials in the GDN ($N=3$ animals, total 6 trials), but the light-off following the end of flicker did initiate a brief depolarization (Fig. 2E, bottom trace). These results are consistent with the previous finding that the magnitude of the light-off response is related to the duration of the preceding illumination.

Responses of the GDN to expanding stimuli

In natural conditions, an approaching predator is signaled by object expansion on the retina, which accelerates as the object nears the subject. In our setup, the looming stimulus expands at a constant speed and could therefore be considered as the last moment of an approaching object. As reported previously (Mu et al., 2012a), an expanding black-square stimulus initiated a depolarization in the GDN, which, in different preparations, showed variable adaptation (Fig. 3A). For example, in the upper trace, only the first expanding stimulus elicited a weak response, after which the neuron was silent to further stimuli of this type. In the lower trace, successive stimulation elected successive responses.

A contracting black square elicits no obvious response, although the membrane potential only returns to its baseline when stimulation ceases. Responses to an expanding white square that provided luminance increase are as distinct, yet as variable, as responses to

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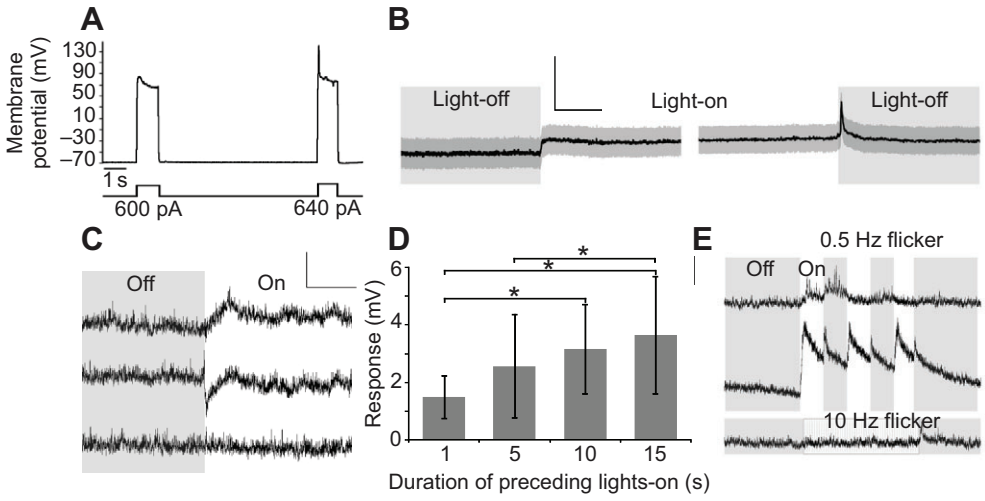

Fig. 2. The GDN responds to current injection and visual stimulation. (A) A depolarizing current injection of 600 pA into the cell body fails to evoke action potentials, but a single spike can be triggered by 640 pA. (B) Average response to light-on and light-off stimuli (*N*=26 flies, *n*=147 trials). Dark gray indicates standard error. (C) Variability of a GDN's response to light-on stimuli: depolarization, hyperpolarization and no response. (D) Depolarization to light-off is larger after longer preceding light-on; here light-off responses after 15 s light-on are compared with 1, 5 and 10 s preceding light-on (ANOVA, *N*=8 flies; *n*=40 trials for 1 s, 38 trials for 5 s, 37 trials for 10 s, 55 trials for 15 s; asterisks indicate $P<0.05$; error bars are ±s.e.m.). (E) Adapting responses to flicker presented at two different frequencies. Scale bars: 2 mV/1 s.

light-on stimuli. Examples shown in Fig. 3B demonstrate weak depolarization in the top two traces, but some hyperpolarization in the bottom trace. Additionally, a contracting white-square stimulus (luminance decrease) also initiated depolarizing responses in some cases (indicated by asterisks, Fig. 3B) but not others. Chessboard square expansion (constant luminance) initiated no obvious GDN responses (*N*=3 animals, total 6 trials). The recording shown in Fig. 3C is of a GDN responding with depolarizations to an expanding black square (top trace) but not to an expanding chessboard square (bottom trace). These results suggest that the efficacious black-square expanding stimulus stimulated the GDN by decreasing luminance, rather than by presenting fast-expanding edges. Faster expansion speeds of the black square were more efficacious than slower ones, with significant differences between responses to high-speed expansion and low-speed expansion (ANOVA, *n*=12 trials in one animal, $P=0.051$ for 40 versus 80 deg s^{-1}, $P<0.01$ for 40 versus 100 deg s^{-1}, $P<0.01$ for 80 versus 100 deg s^{-1}; Fig. 3D). If the neural pathway for the looming response in the GDN is indeed the same as that for the light-off response, the looming-speed recordings suggest that this neural pathway is sensitive to the rate of luminance decrease.

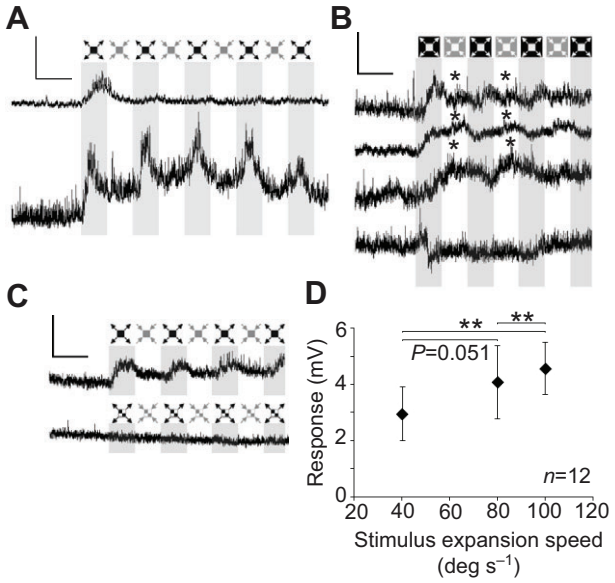

Fig. 3. The GDN responds to expanding stimuli. (A) Two recordings showing different adapting responses to an expanding black-square stimulus. (B) Recordings showing variability of the GDN response to expanding white-square stimuli. Contracting white-square stimuli (luminance decrease) initiate some depolarization (indicated by asterisks). (C) Recordings showing GDN responses to expanding black-square stimuli (top trace) but showing no response to expanding chessboard patterns (bottom trace). (D) Faster expansion of expanding black-square stimuli initiates larger responses (ANOVA, *n*=12 trials in one animal; $^{**}P<0.01$). Error bars are ±s.e.m. Scale bars: 2 mV/1 s.

Responses of the GDN to mechanical stimuli on the antennae

Studies on species of large flies found that the GDN responded to direct mechanical stimulation of the antenna (Bacon and Strausfeld, 1986). This is also seen in the *Drosophila* GDN, which additionally shows subthreshold responses to courtship song (Tootoonian et al., 2012) and to direct antennal displacement (Lehnert et al., 2013). Our results showed that an air puff to the antennae invariably elicited a large EPSP at the beginning of the stimulus (Fig. 4A), but subsequent GDN responses were varied. Stimulus cessation could sometimes also initiate a large EPSP (top trace of Fig. 4A). A sustained depolarization could be elicited through the whole air puff phase (middle trace). Or, an EPSP barrage was seen throughout the wind puff stimulus (bottom trace). An intermittent air puff delivered at approximately 6 Hz produced varying rates of adaptation (Fig. 4B).

Response of the GDN to multimodal (simultaneous visual and mechanical) stimuli

In our experiments, almost all the recorded GDNs showed depolarizing responses to air puff stimuli (28 of the 29 GDN preparations successfully recorded). In contrast, not all of them showed obvious responses to visual stimuli, including both light-off and dark-field expansion. A study of the blowfly *Calliphora erythrocephala* found that the GDN would spike when simultaneously given a mechanical stimulus to the antenna and a light-off stimulus to the eye (Milde and Strausfeld, 1990). Fig. 4C shows an example of *Drosophila* GDN responses to a sequence of individual and combined air-puff and light-off stimuli (with 15 s light-on preceding each stimulus); the response to the combined air puff and light-off stimulus was larger than the response to either the light-off or air-puff stimulus alone. Fig. 4D shows that the intensity of responses to combined stimuli is significantly larger than the response to either the light-off or air-puff stimulus alone ($P<0.01$,

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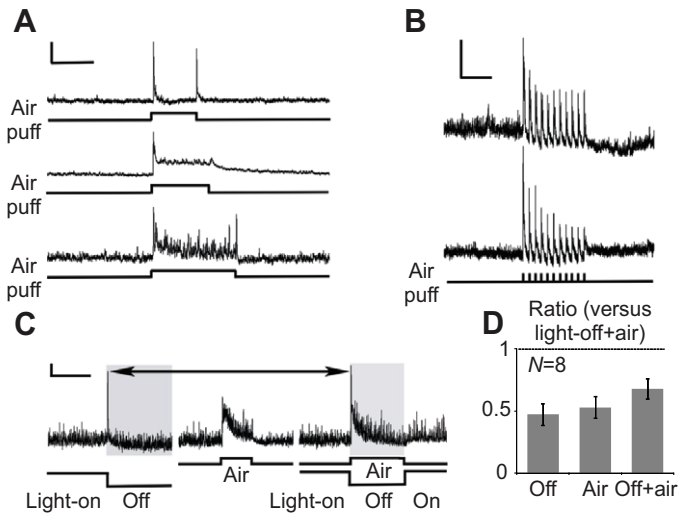

Fig. 4. The GDN responds to antennal displacement and bimodal stimulation. (A) Response to air puffs is variable, although air-puff onset always initiates a large excitatory post-synaptic potential (EPSP). (B) Adapting responses to a train of air-puff stimuli delivered at 6 Hz. (C) Unimodal and bimodal stimuli delivered as a sequence of light-off, air-puff, and simultaneous light-off and air-puff. The double arrows indicate difference of response magnitude between the initial single light-off and bimodal stimulus. (D) Average magnitude of responses to light-off, air-puff, and combined light-off and air-puff stimuli, as a proportion of the linear sum of mean responses to individual light-off and individual air-puff stimuli. Responses to the combined air-puff and light-off stimulus are significantly larger than that to unimodal stimuli (ANOVA, $P<0.01$, $N=8$), but significantly less than their linear sum (ANOVA, $P<0.01$, $N=8$ flies, $n=48$ trials). Error bars are ±s.e.m. Scale bars: 2 mV/1 s.

$N=8$ animals, 48 trials), but was significantly smaller than the linear summation of responses to the individual stimuli (indicated by the dashed line, $P<0.01$, $N=8$ animals, 48 trials).

Multimodal inputs to the GAMDN

Circuit elements

The GAMDN (Fig. 5) provides a useful comparison to the GDN. The GAMDN's cell body, or perikaryon, is equal in size to that of the GDN and its neurite connecting the cell body has the same diameter range (4–5 µm) as that of a GDN (Fig. 5A). The GAMDN is also comparable to the GDN in that its dendritic subfields relate to protocerebral optic glomeruli (Fig. 5D) and to deutocerebral antennal mechanosensory and motor regions (Fig. 5E). In all other respects, these two types of GDNs are distinct. The GAMDN shows no evidence of dye coupling. Unlike the GDN, which has no axon collaterals in the brain, the GAMDN gives rise to extensive beaded collaterals in the brain, indicative of output sites (Fig. 5C,E). These occur in the ipsilateral antennal lobe, the inner antennoglomerular tract, both lobula plates and both AMMCs (Fig. 5E). The axon of the GAMDN extends within the dorsomedial DN tract, where it gives rise to short collaterals in thoracic neuromeres (Fig. 5B). There is no evidence for extensions to motor neuron domains.

Responses

The GAMDN is predominantly a spiking neuron, and thus distinct from the GDN (Fig. 6). It shows a steady resting potential of –55 mV. Abrupt changes of luminance initiate depolarization and sometimes unambiguous spiking responses (Fig. 6A). Typically, membrane depolarization is maintained for several seconds after stimulus cessation. The neuron shows a brief phasic depolarization in response to an air puff onto the antennae (Fig. 6C). The GAMDN's response to looming stimuli (which resulted in depolarization of the GDN) is also variable: either subthreshold depolarization (Fig. 6B) or occasional spikes (Fig. 6D). As in the GDN, those responses to looming might be responses to changes of light intensity rather than to expanding edges.

In summary, tests of this neuron and other smaller DNs (Mu et al., 2012b) demonstrate that DNs other than the GDN respond to bimodal inputs with spiking and subthreshold depolarizations. These results demonstrate the validity of our recording technique, and show that the reluctance of GDN to spike is indeed exceptional.

DISCUSSION

Neural pathways mediating visual inputs to the GDN

Our recordings show that the conditions for eliciting depolarizing responses of *Drosophila* GDN are highly variable. Responses to light-on vary from hyperpolarization to depolarization, contrasting with consistent depolarization to light-off. Anatomical observations suggest that although Col A neurons converging from the lobula contribute a major input to the GDN, there are at least two other channels from the lobula that may contribute to response variability. In larger fly species (Strausfeld and Bassemir, 1983; Heisenberg and Wolf, 1984), electron microscopical observations confirm that these Col A–GDN synapses are monosynaptic, comprising mixed chemical and electrical junctions. If the chemical synapses between Col A neurons and the GDN are excitatory (Enell et al., 2007; Strausfeld et al., 2007), inhibitory response seen in the GDN to some light-on stimuli are likely to derive from a separate neural pathway. Pathways carrying information about light-on and light-off may indeed be interdependent because the intensity of response to light-off is related to the prior duration of light-on. Further recordings from other DNs in larger fly species likewise resolve separate on and off signaling (Gronenberg and Strausfeld, 1992). In the dipteran visual system, motion information is separated at the lamina neuropile into on and off signals, carried by L1 and L2 cells, respectively (Joesch et al., 2010). In large flies, L1 and L2 cells relay via retinotopic interneurons to T4 and T5 cells in the medulla (Strausfeld et al., 2006), as they do in *Drosophila* (Maisak et al., 2013). Conceivably, L1 and L2 also split their outputs to additional parallel channels, the neurons of which penetrate deep into the lobula to levels containing Col A dendrites.

The characteristics of antennal mechanosensory inputs to the GDN

Cobalt-coupling experiments in larger flies (Bacon and Strausfeld, 1986) demonstrated that the GDN is postsynaptic to the terminals of Johnston's organ afferents in the AMMC. The present study also shows that the caudal dendritic branch 4 of the GDN dendritic tree is in apposition to Johnston's organ afferent projections in the AMMC. In larger fly species, cobalt readily passes from antennal nerve afferents into the deutocerebral dendrites of the GDN. This observation is consistent with the recent physiological demonstration that sensory terminals from Johnston's organ make gap junctions with the dendrites of *Drosophila* GDN (Lehnert et al., 2013).

Activation of Johnston's organ receptor neurons in the antenna by the courtship song elicits subthreshold responses in the GDN of *Drosophila* (Tootoonian et al., 2012), as does sound and direct mechanical stimulation of the ipsilateral antenna (Lehnert et al., 2013). Again, neither stimulus elicits spikes. Here we show that the GDN in *Drosophila* also responds to deflections of the antenna by an air puff. The AMMC is divided into five zones, each with different mechanosensory characteristics; zone A neurons respond

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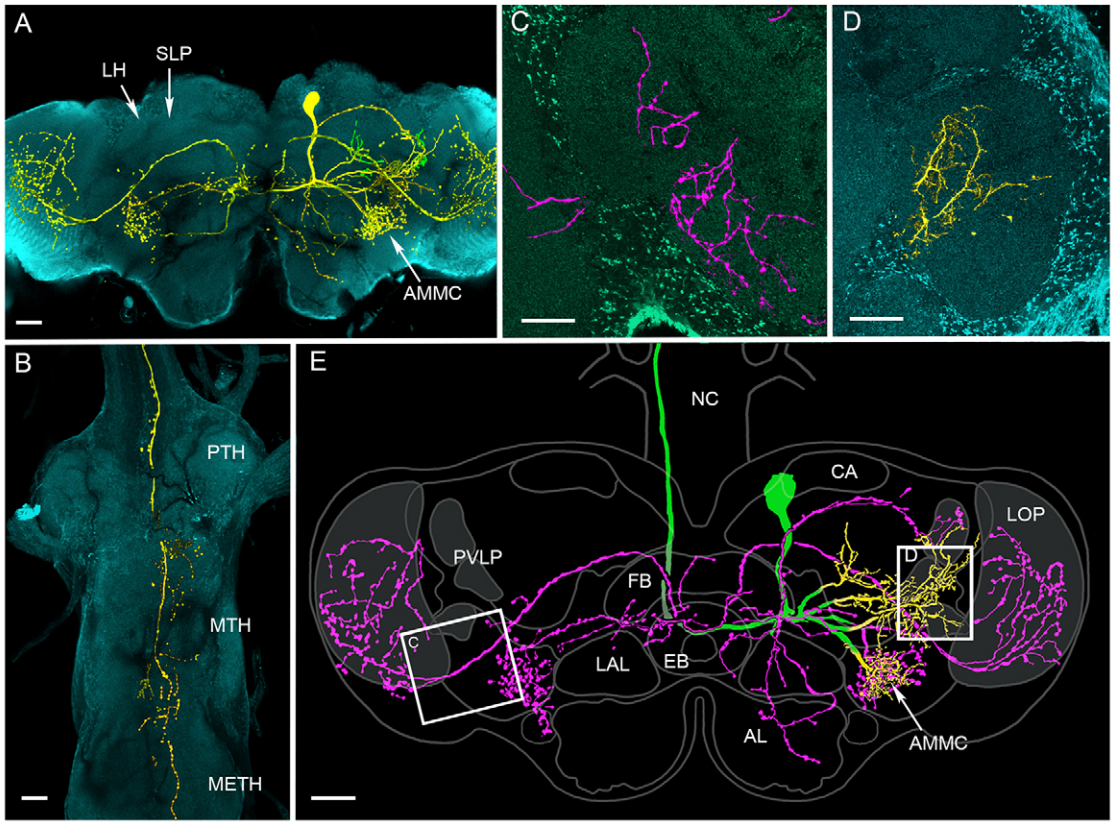

Fig. 5. Scanning confocal micrographs of the giant antennal mechanosensory descending neuron (GAMDN). (A,B) Confocal images of whole brain (A) and thoracic ganglia (B). Note the axon trajectory restricted medially in the thoracic ganglia (PTH, prothoracic ganglion; MTH, mesothoracic ganglion; METH, metathoracic ganglion). Characteristic of this cell type are extended beaded axon collaterals in the brain [some enlarged in C, many of which partially obscure the spiny dendrites (D)]. (E) Color segmentation. Green shows the cell body and its neurite leading to the four primary dendritic branches and axon into the neck connective (NC). Axon collaterals in the brain are shown in magenta; dendrites are shown in yellow. Dendritic domains are well segregated from each other: one in the ipsilateral antennal mechanosensory and motor center (AMMC), and others relating to antero-lateral optic glomeruli in the posterior ventral protocerebrum (PVLP). Together these dendrites reflect the mechanosensory and visual inputs of this neuron. Axon collaterals extend heterolaterally to both lobula plates (LOP), to the ipsilateral antennal lobe (AL), and to beneath the ipsilateral calyx (CA). Boxed areas correspond to C and D. Other abbreviations are: EB, ellipsoid body; FB, fan-shaped body; LAL, lateral accessory lobe; LH, lateral horn; SLP, superior lateral protocerebrum. Scale bars: (A,B,E) 25 µm; (C,D) 10 µm.

to the onset and offset of arista displacement, whereas zone E neurons remain tonically active throughout displacement (Kamikouchi et al., 2006; Yorozu et al., 2009). A previous study shows that one dendrite of the GDN extends to zones A and B (Kamikouchi et al., 2006), which is consistent with the present electrophysiological observations. However, as well as the robust phasic EPSP responses of the GDN at the beginning and end of an air puff, there were also varied tonic components: elevated membrane potentials or increased EPSP activity throughout the air puff stimulus. These observations suggest that the GDN also receives some input from wind-sensitive Johnston's organ afferents projecting to zone E.

The GDN is far more resistant to sensory adaptation in response to repetitive air-puff stimuli than it is to repetitive light-on and -off stimuli. This may be an evolutionary adaptation in that the fly needs to respond reliably to direct potential danger (signified by antennal displacement) but needs to adapt rapidly to a repetitive potential danger (signified by light intensity change). For example, for a fly in a dappled habitat, ambient light intensity is likely to change frequently because of movement of objects in the surrounding environment. Thus, a sudden antennal displacement may be a more sensitive indicator of a looming predator than dimming. Also relevant in this context is that Johnston's organ afferents make monosynaptic electrical synapses with the GDN (Lehnert et al., 2013), whereas there are at least four synaptic delay steps interposed between the retina and the Col A ensemble. The latter circuit offers more opportunity for sensory adaptation. Support for this suggestion comes from physiological recordings in the crab optic-lobe columnar neurons showing adaptation during high-frequency stimulus repetitions, but presynaptic visual neurons responding consistently to the same stimuli (Berón de Astrada et al., 2013).

The interaction between multimodal sensory inputs to the GDN

It is difficult to initiate spikes in the GDN in wild-type red-eyed *Drosophila* by light-off stimuli (Thomas and Wyman, 1984; Levine, 1974). Levine (Levine, 1974) showed that spikes could be generated in mutant flies but only by mechanical stimulation, not by visual stimuli. In larger flies, the GDN will spike only if visual and mechanical stimuli coincide (Milde and Strausfeld, 1990). The present recordings of the *Drosophila* GDN failed to reveal any facilitation between visual and mechanosensory inputs, although the simultaneous pairing of these two danger signals is clearly more likely to induce GDN spiking activity than when either stimulus is presented alone.

The Journal of Experimental Biology (2014) doi:10.1242/jeb.099135

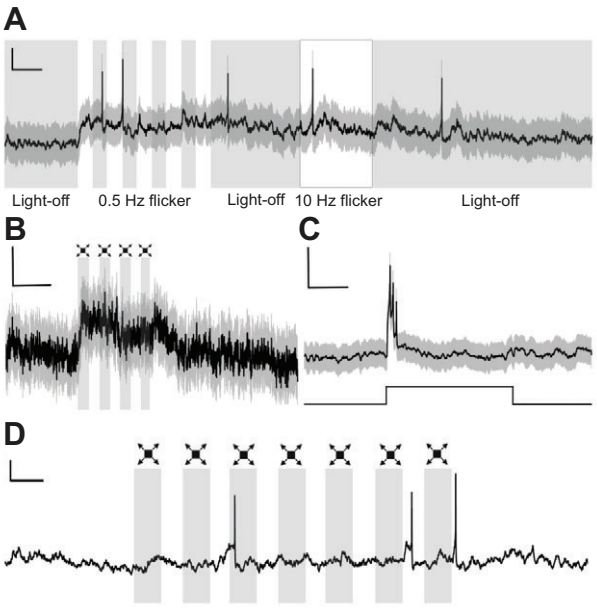

Fig. 6. Spiking responses of the GAMDN to visual and mechanosensory stimuli. Dark gray indicates standard error. (A) Average responses (including both spiking and subthreshold depolarization) to flicker and light-off (*N*=1 animal, *n*=4 trials). The depolarization effect can be maintained for several seconds after the end of the stimulus. Scale bar: 2 mV/2 s. (B) The neuron also shows subthreshold responses to black-square expansion (*N*=1 animal, *n*=8 trials). Scale bar: 1 mV/5 s. (C) Transient spiking response to air-puff stimuli (*N*=1 animal, *n*=8 trials). Scale bar: 2 mV/1 s. (D) Repetitive changes of luminance (expanding black square) trigger depolarization and occasional spiking response. Scale bar: 2 mV/1 s.

The neural pathway for looming-mediated escape behavior

The GDN pathway was long regarded as the quintessential command neuron for visually mediated escape behaviors in flies, but a growing body of evidence has led to a re-evaluation of this view. For example, the GDN either does not spike at all (Fotowat et al., 2009) or only spikes in some trials (Von Reyn and Card, 2012) when presented with a looming stimulus. However, Von Reyn and Card (Von Reyn and Card, 2012) recorded these GDN spikes in a preparation in which the recorded fly could execute a mid-leg 'jump' extension. This implies that the ability of the GDN to spike was dependent not only on sensory stimuli to the head, but also on information provided to it from the level of the mesothoracic ganglion; what that might be is as yet unresolved. Further, Holmqvist (Holmqvist, 1994) found that a visual looming stimulus could trigger escape behavior without activating the GDN in *Musca domestica*. Subsequently it was shown that *Drosophila* undertakes discrete motor actions – raises its wings and coordinates leg movements – before taking off and jumping away from a looming stimulus (Hammond and O'Shea, 2007; Card and Dickinson, 2008a; Card and Dickinson, 2008b; Fotowat et al., 2009). Because these preceding motor actions are not elicited in light-off mediated escape in white-eyed flies, it is likely that an alternative descending neuronal pathway triggers looming-induced escapes.

Why should the GDN spike?

Although in an approximation of the natural sensory environment, our looming stimuli did not elicit a spiking response by the GDN, the neuron could, however, be activated at subthreshold levels by dark expanding stimuli (Mu et al., 2012a). This activation is almost certainly not a response to looming, but to luminance decrease, likely mediated by the same pathway as that for light-off responses. As almost all predator attacks in the natural world would be signaled by sudden luminance decrease and an associated sudden increase in air movement, these or even the latter alone would be sufficient to trigger escape behavior. But if combined luminance decrease and antennal displacement are still not sufficient to initiate a spiking response, could this be due to our restrained preparation suppressing spiking activity, a phenomenon known as restraint-induced inhibition (Krasne and Wine, 1975)?

Recordings from other *Drosophila* DNs (Mu et al., 2012b), exemplified here by the bimodal GAMDN, show that other DNs indeed readily spike. Thus, restraint might not be the reason that the GDN does not spike. Is, then, the quest for its reliable spiking activity that for a mirage? While paired command neurons mediating escape reactions in some species do indeed employ synchronous spikes (Yono and Shimozawa, 2008), this need not be a *sine qua non* of escape circuits in general. Clearly the paired GDNs are part of a most unusual system. In *Musca*, delays between an electrically evoked spike in the GDN and the ensuing response by the tergotrochanteral muscle is approximately 2 ms, suggesting that only a single chemical synapse intervenes between the GDN and the effector (Bacon and Strausfeld, 1986). This is substantiated by the passage of dyes from the GDN, indicative of electrical junctions. These characterize reciprocal electrical synapses between both GDN terminals in the mesothoracic ganglion as well as between each terminal and the bilateral pair of PSIs and the TTMNs. In the brain, dye coupling resolves the GDN as electrically contiguous with Col A neurons. Thus, non-spiking responses are likely to be relayed virtually unimpeded to the leg extensor muscle and to the PSI, which is chemically presynaptic onto the axons of wing-depressor motor neurons. That both the left and right GDNs are electrically coupled to each other at their terminals, and that both are electrically coupled to the giant contralateral interneurons in the brain, reveals a system that may have evolved to transmit analog signals rapidly and symmetrically to bilateral motor outputs. Therefore, a plausible explanation for this non-spiking aspect of the GDN is that its function is to prime interneuron–motor neuron circuits that only reach spike threshold in response to coincident input from the GDN and from local thoracic mechanoreceptors. There is a precedent for the importance of local sensory feedback combining with descending information to provide appropriate motor actions. A study on the maintenance of stabilized flight by flies has shown that although visual information relayed by DNs conspires with information from sensory circuits to provide rapid adjustments of motor output, it is the local sensory feedback, and not the descending pathways, that dominates this interaction (Sherman and Dickinson, 2004).

Finally, it should be recalled that the GDN does not function in isolation. Indeed, anatomical studies on larger flies showed that the GDN belongs to a cluster of DNs, all of which receive visual inputs from the lobula (Strausfeld and Bacon, 1983; Strausfeld et al., 1984; Milde and Strausfeld, 1990). It is possible that the DNs in this cluster process a variety of visual primitives, including looming, and that they comprise a system supplying the thoracic motor centers with parallel pathways that mediate various locomotory behaviors including flight initiation that are quite distinct from that triggered by the GDN circuit.

MATERIALS AND METHODS

Terminology

The term 'giant fiber' is a general one, used to denote particularly wide-diameter axons. Here the name 'giant fiber' has been substituted by the

moniker 'giant descending neuron' (GDN) to bring the present description into line with previous studies of the largest DNs in the brains of other dipterous species. Names and abbreviations used to describe brain regions follow established nomenclature (Ito et al., 2014).

Flies

Drosophila melanogaster were raised on standard cornmeal-agar medium under a 12 h:12 h light:dark cycle. The experimental flies were 2- to 4-day-old adult female *Drosophila melanogaster* of the UAS-mCD8::GFP A307 line (for GDN), or the progeny of crossing GAL4 enhancer-trap lines, FBst0006488 (Bloomington *Drosophila* Stock Center) (for GDN), homozygous A307 (for GAMDN) and NP5092 (for GDN and Col A), with a UAS-GFP reporter line, UAS-GFP S65T.

Revealing afferent inputs to the GDN

To examine the convergence onto the GDN by Johnston's organ afferents, the distal segment of an antenna was removed, and the tip of the broken second antennal segment (scapus) was threaded into the tip of a broken glass electrode filled with dextran-conjugated Texas Red solution (3000MW, Invitrogen, Grand Island, NY, USA). Fills into the receptor axons were of 2 h duration, after which whole-cell patch-clamp recording of the GDN culminated in filling it with biocytin, which was subsequently labeled with Streptavidin:Cy3. Using a method described previously by Lin and Strausfeld (Lin and Strausfeld, 2012), after they had been fixed in buffered paraformaldehyde and washed in phosphate buffer, brains were dehydrated, embedded in Spurr's resin (Electron Microscopy Science, Hatfield, PA, USA), sectioned at 20 μm and then mounted in Permount (Fisher Scientific, Fair Lawn, NJ, USA) under thin glass coverslips. Serial sections were scanned with a Zeiss Pascal three-line confocal microscope at increments of 1 μm.

The convergence of visual inputs onto the GDN was resolved in the F1 progeny of crossing the GAL4 line NP5092 with a UAS-GFP reporter line, UAS-GFP S65T, which generated GFP-labeled Col A neurons. The GDN in these F1 progeny was subjected to whole-cell patch-clamp recording and dye filling before the brain was fixed and labeled with antibodies raised against GFP as described in Mu et al. (Mu et al., 2012a), and then embedded in plastic as described above. Brains were serially sectioned and the GDN and Col A neurons were reconstructed using scanning confocal microscopy to resolve convergence of Col A axons onto GDN dendritic arbors. UAS reporter lines resolved Col A neurons contiguous with the giant interneurons. These lines were treated with anti-GFP as above and then counter-labeled using antisera raised against β-tubulin.

Electrophysiology and experimental stimuli

Detailed methods regarding animal preparation, whole-cell patch-clamp recording from the identified GDN and GAMDN cell bodies, and subsequent immunohistology were the same as described in Mu et al. (Mu et al., 2012a) except for some changes in experimental stimuli. Visual stimuli were presented by a customized flat LED arena (Reiser and Dickinson, 2008) composed of 8 × 7 LED panels (Mu et al., 2012a). Stimuli were: full-field flicker; an expanding (looming)/retracting (receding) square black block on a bright background (40, 80 and 100 deg s^{-1}); a square expanding/contracting bright block on a black background; and a chessboard block on a bright background with a total constant luminance during expansion and contraction. An aluminum tube (~1 mm diameter), connected to a compressed air source, was fixed in front of the animal's head, approximately 1 cm away from the antennae. A Grass S48 stimulator (Astro-Med Inc., West Warwick, RI, USA) and a solenoid valve (Parker Hannifin Corp, Tucson, AZ, USA) were used to divert the airflow from an open channel to the tube facing the head of the animal. The wind speed was approximately 2 to 8 mm s^{-1}.

Data analysis

The resting potentials of the GDNs recorded in our experiments were similar (−68.4±4.5 mV, mean ± s.e.m., N=36). The response to each stimulus was defined as the difference between the maximum membrane potential during the stimulus and the mean membrane potential in the 500 ms before the onset of the stimulus. To compare neuronal responses under different stimulus conditions, one-way repeated-measures ANOVAs were conducted using stimulus condition as the sole factor. If a significant effect was found, multiple comparisons among pairs of conditions were conducted using the Bonferroni correction. All P-values reported for multiple comparisons were Bonferroni corrected.

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Competing interests

The authors declare no competing financial interests.

Author contributions

All authors contributed to the design of the experiment. L.M., J.P.B. and N.J.S. wrote the manuscript. L.M. performed the experiments and physiological analysis. N.J.S. undertook anatomical analyses.

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